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14. ABSTRACT
The primary research focus of the San Diego State University (SDSU) Structural Biochemistry Laboratory led by Dr. Tom Huxford is to understand how the assembly of macromolecular complexes gives rise to fundamental healing processes such as inflammation in response to trauma or infection and the repair of damaged muscles. The goal of this proposal was to secure funds for the acquisition and implementation of a NanoTemper Technologies Monolith NT.115 Thermophoresis instrument. The instrument, which is one of the first of its kind to be acquired and implemented within the state of California, permits rapid and accurate measurement of equilibrium binding

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Tom Huxford
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Report Title

Final Report: Measuring Equilibrium Binding Affinity of Biological Macromolecules in Solution by Thermophoresis

ABSTRACT

The primary research focus of the San Diego State University (SDSU) Structural Biochemistry Laboratory led by Dr. Tom Huxford is to understand how the assembly of macromolecular complexes gives rise to fundamental healing processes such as inflammation in response to trauma or infection and the repair of damaged muscles. The goal of this proposal was to secure funds for the acquisition and implementation of a NanoTemper Technologies Monolith NT.115 Thermophoresis instrument. The instrument, which is one of the first of its kind to be acquired and implemented within the state of California, permits rapid and accurate measurement of equilibrium binding affinities of biological macromolecules in solution. This qauntitative information plays a vital role in supporting the static structural data that researchers in the lab typically obtain through x-ray crystallography. Thus far, the instrument has been used to measure dimerization, DNA binding, and co-activator binding by transcription factor NF-kappaB binding. Initial studies have also begun that are attempting to measure the interaction of myosin motor proteins with the muscle assembly and repair chaperone protein UNC-45.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Cathrine Aivati	0.00	
Matthew Mekany	0.00	
Samantha Cohen	0.00	
Christy Milani	0.00	
FTE Equivalent:	**0.00**	
Total Number:	**4**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Tom Huxford	0.00	No
FTE Equivalent:	**0.00**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Linda Honaker	0.00	Chemistry
Jeffrey Noblet	0.00	Chemistry
Lisa Acuna	0.00	Chemistry
Perla Pena Palomino	0.00	Chemistry
FTE Equivalent:	**0.00**	
Total Number:	**4**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 3.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 3.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 1.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 1.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 1.00

Names of Personnel receiving masters degrees

NAME
Christy Milani
Total Number: **1**

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

We purchased the Monolith NT.115 MicroScale Thermophoresis instrument from NanoTemper Technologies in March, 2014 and installed it in April, 2014. This instrument enables the rapid determination of equilibrium binding affinities between macromolecules in solution. The technological basis for the measurement is that large compounds display characteristic rates of movement along a temperature gradient in solution. Changes in the molecular weight, charge, or hydration shell will affect the thermophoretic mobility of the compound. Therefore, changes in thermophoresis of a fluorescently labeled compound can be monitored upon titration with a binding partner and will correlate directly with the concentration at which the binding partner forms an association. Two radiation sources, one infrared laser that carefully controls a small gradient in solution temperature and one LED that provide excitation frequencies to trigger fluorescence in the tagged compound, function simultaneously to treat solution samples that have been introduced into a capillary for analysis. The capillaries hold between 3-5 micrometers of total sample. Therefore, Thermophoresis provides a rapid means for direct measurement of molecular interactions in a true solution environment using very small volumes of sample. Furthermore, by optimizing the capillary coatings to minimize sample aggregation, one can control the reaction environment so as to negate surface binding effects.

The first experiments that we performed with the new instrument were a confirmation of the technique and the instrument by measuring the interaction of a small molecule (AMP) to an aptamer AMP-binding DNA. This experiment was carried out using control reagents acquired from NanoTemper Technologies and yielded clean data that agreed well with the published literature values. We next attempted to measure binding affinity of NF-kappaB p50 homodimer to fluorescently-labeled kappaB DNA from the promoter of the IL-6 gene (IL-6 kappaB), from the intronic enhancer of the immunoglobulin kappa light chain gene (Ig kappaB), and a scrambled version of the Ig kappa DNA. The fluorophore that we selected was Cy5, as it works well with red and green filter set that we had installed in our instrument. We were not able to make measurements of the binding. This caused us several months of trouble shooting as we tested different parameters such as DNA concentration, the addition of poly-dI/dC as a non-competitive binding DNA mimic, detergents, different capillary surfaces, temperature, etc. We carried out a parallel study in which we switched from using long (41-mer) double-stranded deoxy-oligonucleotides to shorter (13-mer) versions. This did not affect our results.

The solution that we finally arrived upon was to label the NF-kappaB p50 protein and use unlabeled kappaB DNA. We first used fluorescent labeling kits that attach Cy5 labels to free lysines in proteins. This method worked reasonably well and we routinely calculated our labeled sample to contain roughly 1 label per four proteins. Thermophoretic measurements employing these labeled p50 proteins allowed for determination of DNA binding in the 100 nM range. However, labeled sample quality was irreproducible and the fluorescence signal we observed in our labeled p50 proteins decreased steadily over time.

In order to improve upon these limitations, we next designed, engineered, expressed, and purified a nearly full length murine NF-kappaB p50 subunit protein as a fusion with a C-terminal Green Fluorescent Protein (GFP). This protein has been prepared now two times and thus far has proven much more reliable in terms of reproducibility of sample behavior and binding measurements. On the basis of this success, we currently have a series of binding studies planned. These include measurement of dimerization affinity of the NF-kappaB p50:p50 homodimer and p50:p65 heterodimer, the binding affinity and cooperatively of NF-kappaB p50:p50 homodimer to different kappaB target DNA, and the interaction of the nuclear IkappaBzeta with NF-kappaB p50 homodimer on DNA. All of these values have been measured previously by gel shift assays, analytical ultracentrifugation, fluorescence polarization, and surface plasmon resonance spectroscopy. However, the values differ significantly and it will be valuable to be able to make all the measurements using one solution-based technique. The IkappaBzeta:NF-kappaB:DNA interaction is completely novel and will provide new data within the context of NF-kappaB-driven transcriptional complex formation on DNA. We look forward to completing this study in the coming months and reporting the results in a manuscript.

In other projects, we have begun analyzing interactions between the myosin chaperone UNC-45 and myosin using UNC-45 proteins that are labeled via kit. The result of these experiments was not very convincing and so, in light of our success with labeling the NF-kappaB p50 subunit protein, we have recently generated our myosin chaperone protein UNC-45 as a fusion protein with GFP. We are preparing this protein to enable measurement of binding affinity with myosin motor domain proteins purified from chicken breast and from whole flies. As a positive control, we will measure the interaction of Drosophila GFP-UNC-45 with the Drosophila Hsp83 protein (the fly homolog of Hsp90). This protein has been shown to interact specifically, though with relatively low affinity, to the TPR domain of UNC-45. Once we have measured myosin and Hsp83 binding affinity to UNC-45 separately, we will determine whether there is cooperativity upon binding to both partners simultaneously. Our future plans are to use the x-ray crystal structure of Drosophila UNC-45, which we reported in 2011, as a template for structure-based deletion and mutagenesis of the protein in order to map the interaction surfaces that mediate interaction of UNC-45 and myosin.

Finally, we are also working toward measuring the affinity of association of the IkappaB Kinase 2 subunit. Our x-ray crystal structure, published in 2013, suggests that the dimerization interface in homodimers of human IKK2 is much less extensive than previously supposed from x-ray crystallography on the inactivated Xenopus enzyme. Using approaches similar to what we described for NF-kappaB p50 homdimer, we will assess the binding affinity of IKK2 subunit homodimers and test for the ability of peptides designed to target the dimer interface to disrupt binding affinity. In support of this work we have engineered and purified human IKK2 subunit proteins. We are working on a best approach for labeling and making measurements via Thermophoresis.

Technology Transfer